CLXXXII. ELECTROPHORESIS OF SERUM GLOBULIN

II. ELECTROPHORETIC ANALYSIS OF NORMAL AND IMMUNE SERA

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In an earlier paper [Tiselius, 1937, 1] the electrophoretic mobilities of different preparations of serum globulin from horse and rabbit sera were measured over a *p*H range including the isoelectric point. It was concluded, *inter alia*, that none of the preparations studied represented a homogeneous substance as judged by electrochemical properties. By subjecting serum to prolonged electrophoresis, and collecting the slowest fractions, a serum globulin material was isolated which was different from those obtained by the common methods and more homogeneous than these.

An entirely new construction of electrophoresis apparatus has now been made, with the special purpose of studying the serum proteins, but of a very wide applicability for elucidation of the constitution of mixtures of proteins and other high molecular substances and for isolation of the components of such mixtures in pure form, a procedure which may be described as electrophoretic analysis. The apparatus and the procedure have been described in detail elsewhere [Tiselius, 1937, 2], so that only the main principles in the new construction need be given here.

The heat convection currents, which form the most serious source of error in systems of as high conductivity as serum and serum proteins, have been largely eliminated by having the apparatus working at $+4°$, at which the density of water is a maximum, and also by using a U-tube built of flat cells, with a rectangular cross section. By these means 7–10 times as high voltage as in the earlier apparatus could be used, with a corresponding increase in separation capacity or "resolving power" in the study of mixtures. Instead of the ultraviolet photographic method earlier used for observation of the migrating boundaries in the U-tube, a method depending upon refractive index (Toepler's "Schlieren" method) is applied [*v.* also Tiselius *et al.* 1937]. With this method, each boundary appears as a black band in the image of the U-tube in the focus of the camera. Components of concentrations as low as a few hundredths of a per cent may thus be observed. The electrophoresis apparatus is shown in Fig. 1. The U-tube sections can be moved with respect to each other so as to cut off the column of solution into four parts, for analysis or for isolation of pure components. This movement is effected by a pneumatic arrangement (dotted in

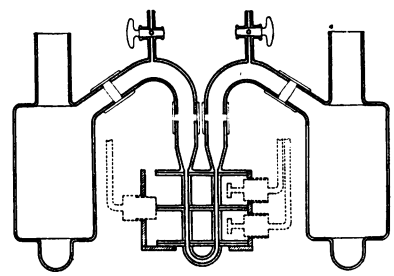

Fig. 1. Apparatus for electrophoretic analysis.

the figure). On the right and the left are the tubes for the reversible electrodes, with a large volume of buffer solution between the electrodes and the U-tube. Fig. 2 shows a photograph of the boundary of a homogeneous protein (ovalbumin) after migration through about half of the length of one compartment. If one wishes to separate two components, which migrate with somewhat different velocities, but in the same direction, both components may have to migrate out of the U-tube before any appreciable separation has been obtained. This difficulty is overcome by the following arrangement. A cylindrical ebonite rod, slowly sinking down into or lifted out of one of the electrode tubes by means of clockwork, causes a slow and uniform movement of the solution as a whole through the U-tube, at any desired rate, in the opposite direction to the electrophoretic migration. With proper adjustment, one component will migrate to the right with the same "apparent" velocity as the other component to the left, and at the end of the experiment each half of the U-tube will contain only one component in the pure state. This procedure, whereby the separation capacity of the apparatus can be used to its full extent, has been found extremely useful in work with the serum proteins.

In investigations of this kind it is necessary to have the same buffer medium throughout the apparatus. Therefore the protein solutions were dialysed against a large volume of the buffer solution to be used as supernatant.

Fig. 2.

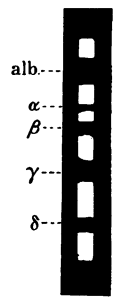

Fig. 3*a*.

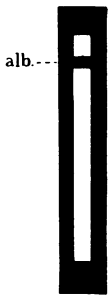

Fig. 3*b*.

Fig. 2. Photograph of the migrating boundary of a homogeneous protein (crystallized ovalbumin).

Fig. 3*a*. Photograph of the migrating boundaries of horse serum after 80 min. at 7·25 V. per cm.

Fig. 3*b*. Photograph of the migrating boundary of serum albumin, isolated from serum by electrophoresis. Same time and voltage as in Fig. 3*a*.

The first experiments with the new apparatus showed very clearly that different fractions of serum globulin, prepared by precipitation, as well as samples of whole serum, consisted of several components, migrating at distinctly different rates. Fig. 3*a* shows the behaviour of 4 times diluted horse serum in the apparatus. The serum had been dialysed overnight against the buffer used (163·5 ml. $M/5$ Na_2HPO_4 + 10·0 ml. $M/5$ NaH_2PO_4 per l., pH = 8·03, ionic strength = 0·1). The potential gradient in the U-tube was 7·25 V. per cm., and the exposure was taken after 80 min. A compensation movement of 7·9 mm. per hr. during 95 min. in the same direction as the migration was used to bring out the slowest moving boundary, which otherwise would have been masked by the glass plate at the bottom of the cell. See also the illustration in the previous publication [Tiselius, 1937, 2; Fig. 4]. For comparison Fig. 3*b* shows the result obtained with serum albumin (isolated by electrophoresis) under the same conditions.

It is especially important to notice the symmetry of the phenomenon, since disturbances due to thermal convection or boundary anomalies may give rise to several boundaries, which, however, are never alike on both sides. The conditions of the experiment were chosen so that the risk of such disturbances were minimized. As mentioned above, uniform and well-defined proteins like crystalline egg albumin when studied under the same conditions give only one boundary on each side. The most convincing proof that it is a question of a real separation into different components is given by the experiments described below, in which it was possible, by suitably conducting the electrophoresis, to isolate from horse serum four different fractions which can be observed optically and to study the mobility-pH relationship for each component separately.

The fastest of these components could be identified with serum albumin. The other three are found in varying amounts in all serum globulin preparations investigated, and are more or less completely precipitated by half saturation with ammonium sulphate. They will therefore be named α, β, and γ serum-globulin. It has not yet been possible to isolate a protein corresponding to the slowest migrating boundary. As will be shown below, this substance (which will be called the δ-component) is formed of the others in varying amounts, depending upon the concentration. The migration of the δ-boundary in the serum can be observed only on the positive side: it is very marked in concentrated solutions.

Electrophoretic analysis of several different samples of normal sera from man, horse and rabbit as well as of immune sera gave similar pictures, although the relative amounts of the components varied considerably. It is therefore a phenomenon of very general importance.

The conditions of the electrophoresis experiments as a rule differ, according to whether mobility determinations or preparative separations are the main purpose. In the former case one should work with low protein concentrations in order to avoid boundary disturbances. It is not possible, therefore, to make mobility determinations on undiluted serum (*v.* p. 1470). For separation purposes, one usually wishes to have the concentration as high as possible, in order to get larger amounts of material. In this case the migration of the boundaries is usually markedly altered, and the different sides of the U-tube do not give concordant results. The optical observation of the boundaries, however, makes it possible to locate the components in the tube in the course of the separation, and serves as a guide for the proper choice of voltage and rate of compensation.

It should be emphasized that in many cases the optical-electrophoretic analysis also may serve as a guide in seeking for suitable methods of fractionation, other than by electrophoresis. We have already had several examples of this in our laboratory. Electrophoretic diagrams, such as those in Fig. 3, give us very complete information of the composition of any fraction prepared, for example by precipitation methods. As will be shown below, one obtains not only qualitative information of which components are present but also a quantitative estimate of their relative amounts. Some serum protein fractions prepared by ammonium sulphate precipitation in the usual way were studied according to this method. The results demonstrated that the precipitate obtained by 30% saturation contained appreciable amounts of the fastest component (serum albumin), whereas the proteins which remain in solution after precipitation of serum with 55% saturation contained 25% of the globulin fractions, all of which were present. This illustrates the extremely high tendency to co-precipitation which is well known to all workers with the serum proteins. However, the pseudoglobulin prepared by electrodialysis proved to contain as

much as 85% of the fastest globulin component (globulin α) and 15% of the slower globulin γ, and none of β or δ. As globulin α is rather difficult to obtain in sufficient amount by direct electrophoresis of serum (its concentration in serum being rather low), pseudoglobulin was found to be the best starting material for preparation of larger amounts of globulin α, for there is an appreciable difference in the mobilities of α and γ and the intermediate β is absent, and so preparation of α by electrophoresis of pseudoglobulin is very easy.

The present paper is intended to describe the electrophoretic analysis of serum, the isolation of pure fractions and an investigation of some of their physical properties. The differences in chemical properties will be the subject of a forthcoming publication.

Electrophoresis of whole serum at different p*H values and constant ionic strength*

The sera (kindly furnished by Statens Bacteriologiska Laboratorium, Stockholm) were from normal horses. In this work no preservative was used in the sera or in the purified fractions. Toluene causes precipitation. Freezing in a refrigerator at −8° to −10° does not seem to change the properties of the solutions and was always applied, unless otherwise stated. The samples were usually diluted 4 times with the buffer to be used and dialysed against this buffer in the cold, until there was no appreciable difference in *p*H inside and outside.

The experimental results are seen from Table I and the curves in Fig. 4.

Table I. *Electrophoretic mobilities of the components in 4 × diluted normal horse serum at different* p*H values and constant ionic strength 0·1. Temperature ±0·0°*

		Mobility cm.^{2} V.$^{-1}$ sec.$^{-1}$ × 10^5				
Buffer	*p*H	Albumin	Glob. α	Glob. β	Glob. γ	Glob. δ
Acetate	5·05	−1·39	−0·36	−0·2	+1·02	+1·53
Phosphate	6·02	−4·24	−2·76	−2·10	−0·23	+0·4
,,	6·87	−5·39	−3·98	−3·25	−1·20	−0·2
,,	8·03	−7·61	−5·79	−4·57	−1·90	−0·1

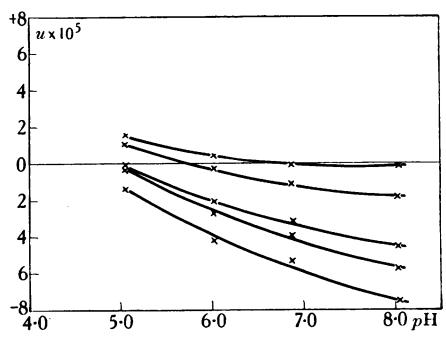

Fig. 4.

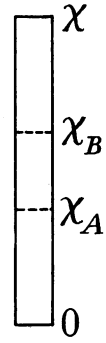

Fig. 5.

Fig. 4. The mobilities of the different components in 4 times diluted horse serum at different *p*H values and constant ionic strength (0·1).

Fig. 5. Determination of the amounts of two components from the electrophoretic diagram.

Experiments were also made at more acid *p*H values. It is, however, difficult to identify with certainty all the boundaries in unfractionated serum near the isoelectric point; compare Fig. 7, giving the mobilities of the purified fractions.

Serum, even after dilution with buffer, showed a more or less marked opalescence, but the albumin and the albumin + globulin α layers were always quite clear, with a yellow colour. The opalescence always migrates with the β-boundary. The γ-boundary is usually more diffuse than the others, indicating that this component is less homogeneous.

The interesting question of how non-protein constituents of serum migrate in electrophoresis will be investigated in the future. In this connexion reference may be made to the important work on this subject by Bennhold [1932] and Pedersen & Waldenström [1937], who were able to show that the yellow pigment bilirubin migrates with the albumin. We were able to confirm this observation. It should be noted, however, that Bennhold's globulin boundary corresponds only to the globulin β in the present work, which is the most easily visible on account of the opalescence. The interesting conclusions of this author, as far as serum globulin is concerned, therefore do not refer to whole globulin, but only to one fraction. Measurements of the migration of cholesterol in serum have been made by Mellander [1935], using Theorell's apparatus.

One gets the impression that the β-globulin is somewhat more coloured than the solution above it, but on account of the strong opalescence in that fraction it is not easy to decide whether this is true or not. When precipitating serum by 55% saturation with ammonium sulphate, the remaining solution contains mainly albumin, but also all the other components, although in much lower amount. The opalescence of β has now disappeared, and it can be seen clearly that this component carries part of the yellow colour. Whether this is bilirubin or another pigment has not yet been decided.

Some orientation measurements on the distribution of carbohydrate after electrophoresis were made by means of the method of Tillman & Philippi [1929]. A solution containing equal parts of mannose and galactose was used as a standard, but the main purpose of the analysis was to make relative determinations. The following values were obtained for the different fractions, in per cent carbohydrate of the total amount of protein: serum albumin 1·4%, globulin α 0·4%, globulin β 2·2%, globulin γ 0·7%. The original serum had 2·2%. It is seen that the fractions contain less carbohydrates so some has evidently been removed in the separation. It is quite possible that further fractionation would remove still more. The amounts obtained were not sufficient for a repeated electrophoresis without diluting the samples too much.

The electrophoretic analysis can be arranged so that the relative amounts of the components can be estimated. This application of the method has not yet been worked out completely and will be dealt with in another publication. If there are only two components, a determination of their relative amounts is made most accurately by reading the positions of their respective boundaries after a suitable separation (χ_A and χ_B, Fig. 5). If the total length of the compartment containing both boundaries is χ, one obtains

$$\chi_A c_A + \chi_B c_B = \chi c^1,$$
$$c_A + c_B = c,$$

where c_A and c_B are the concentrations of components A and B in the original solution of total concentration c, and c^1 is the total concentration of material in the compartment in question, as determined by analysis of the contents after electrophoresis. By combination of these two equations c_A and c_B are obtained. This method is convenient and accurate, as the positions of the boundaries are obtained with high precision. It can be applied also to three components if by suitable compensation (see above) one boundary is kept in the compartment

below. By analysing the total amount in both compartments, together with the original solution, we get three equations for determination of the three unknown concentrations.

It is not possible to apply this procedure to serum with its 5 boundaries. Instead the concentrations were determined by a purely optical method: a concentration gradient integration analogous to that used in the computation of ultracentrifuge sedimentation diagrams, but somewhat simplified [*v.* Tiselius, 1937, 2]. Whichever method is used it is essential that the conditions of disturbance-free electrophoresis be rigidly maintained. Determinations of this kind are possible only in solutions of low protein concentration, in the presence of large amounts of salts. Also there should be no mutual interaction between the different components. Under these conditions the integration gives the same result in both limbs of the U-tube. The following concentrations were found for serum, diluted 10 times, in phosphate buffer of *p*H 8·03 and ionic strength 0·1.

	Positive side %	Negative side %	Average %	Rel. conc. %
Albumin	0·280	0·270	0·275	35·6
Globulin α	0·098	0·092	0·095	12·3
,, β	0·156	0·135	0·145	18·7
,, γ (+δ)	0·238	0·280	0·259	33·4

Methods like the latter require a high degree of optical perfection in the glass windows of the thermostat and the cells. So far only estimations of concentrations have been made in this way, but attempts will be made to improve the accuracy. For orientation experiments in studying the variations in kind and proportions of the components of different sera the procedure is very convenient.

A comparison was made according to this method of the relative concentrations of the components of (1) a normal rabbit serum, (2) a serum from the same animal, immunized with a solution of 4 times recrystallized egg albumin. For these sera the author is much indebted to Dr M. Heidelberger. The antiserum was highly potent: about 25 % of the total nitrogen was specifically precipitable with egg albumin. Optical analysis gave:

	Normal serum %	Antiserum %
Albumin	66·2	51·2
Globulin α	6·6	7·4
,, β	10·0	8·4
,, γ (+δ)	17·2	33·0

Thus there is a very great increase in the slow fraction. Subsequent isolation of this by electrophoresis gave a preparation which was specifically precipitable to an extent of 85 %. The other fractions did not give any precipitate with egg albumin solution.

This method of studying immune sera, and of concentrating antibodies seems to be of considerable interest [*v.* also Tiselius, 1937, 3]. The beautiful methods of Felton [1931; 1932] and of Heidelberger & Kendall [1936], applied to antipneumococcus sera for isolation and purification of antibodies by dissociation of specific precipitates, are not so successful with anti-egg albumin sera. Work along these lines with different antisera is being continued. The fact that the antibody properties are carried only by one of the observed globulin fractions is strong support for the view that these fractions show essential differences in their chemical and physiological properties.

The experiments referred to in Fig. 4 above were all made with 4 times diluted serum. In a number of other experiments the concentration was varied from undiluted to 10 times diluted serum, all in the same phosphate buffer of *p*H 8·03 and ionic strength 0·1. The phenomenon was essentially the same at all concentrations. In undiluted sera the boundary disturbances are very marked: conductivity measurements show that the buffer becomes more dilute at one side and more concentrated at the other. Effects of this kind develop as a result of the influence of the high protein concentration on the transport numbers of the buffer ions. Mobility measurements cannot therefore be made. Sometimes there is an indication of one more boundary just behind the albumin, but it does not seem justifiable to consider this as evidence of the existence of another component, under the rather ill-defined conditions prevailing at the boundary between undiluted serum and pure buffer solution.

There is, however, one marked change that takes place when passing from the dilute to the concentrated solutions. The δ component increases at the cost of the other globulins. In order to study this effect, and also to get more detailed information regarding the conditions in concentrated sera, the following procedure was used with the object of eliminating as far as possible the boundary anomalies. Since these depend only on the differences in concentration, one may study the migration of the components of very nearly undiluted serum, or any concentration, by using a somewhat more dilute serum as supernatant in the upper compartments of the U-tube. An experiment with 1/2 serum against 1/4 serum gave very nearly the same results as the 4 times diluted serum studied previously. Only the δ-component was stronger and appeared on both sides. Undiluted serum against 6/7 serum showed the effect very markedly: only the albumin and the δ-boundary appeared, with faint indications of the other three.

It is not easy to give an entirely satisfactory explanation of this rather puzzling phenomenon. There is, however, little doubt that the effect is real, since boundary anomalies should not appear under the last-mentioned conditions (which have been controlled by experiments with other protein mixtures). Evidently the charge of the globulins in undiluted serum is almost completely neutralized by some unknown substance, which must carry a positive charge even at *p*H 8. This neutralizing agent on dilution is dissociated off more or less completely, and the globulins α, β, γ, are then able to show their individual migration. Such an effect would give rise to a well-defined boundary on the positive side, but to a markedly spread-out boundary on the other, in agreement with observation. If this view is correct, the substance would be found (possibly very much diluted) behind the slowest component on the negative side. So far tests for proteins, amino-acids, carbohydrate, glucosamine have been negative; possibly the concentration is very low.

However this may be, the fact that serum globulin in undiluted blood serum is electrically neutral, and acquires a charge first on dilution, is of considerable interest. It should also be noted that the sedimentation properties of the protein components in whole serum change strikingly with the concentration [McFarlane, 1935].

Very marked changes are observed in the electrophoretic diagrams of serum which has been "thermo-inactivated", i.e. heated to 56° for 10 min. The serum albumin boundary suffers a very large reduction in its mobility and migrates near the β-globulin. A similar change was observed in a serum which had been extracted with alcohol-ether at low temperature to remove lipoids (kindly supplied by Dr E. Holiday). Possibly this is an indication of incipient

denaturation. Otherwise the diagrams obtained from fresh or frozen sera of different horses were quite reproducible, although fluctuations in the relative amounts were often noticed.

Isolation of the protein components

The complete fractionation by electrophoresis of a mixture containing 4 components is not an easy matter. The capacity of the apparatus is rather small, and if a separation has to be made in two steps, the solution becomes rather dilute. All fractionation was made at *p*H 8·03, since the mobility differences are largest at alkaline reaction.

Serum albumin is easily obtained in a concentration of 2–3 % by adjusting the compensator so as to give globulin α an apparent mobility of zero, using undiluted serum. Then the upper positive compartment will contain pure albumin, and by observing the boundaries the separation can be controlled easily. One should not let any of the slower boundaries pass through the bottom of the U-tube in the fractionation. This upsets the hydrostatic equilibrium in the tube and gives rise to convections, which may cause the formation of quite sharp boundaries, falsely indicating new components. For this reason it is preferable first to isolate a considerable volume of the two fastest and the two slowest components, and then run the resulting fractions again to complete separation. In electrophoretic purification, in contrast to other methods, the preliminary removal of part of an impurity does not offer any advantage (unless there is mutual combination). It takes just as long a time to prepare pure albumin from a mixture containing only albumin + globulin α as if one starts with whole serum. There is even evidence for the statement that a large amount of impurity may be of advantage: it gives a sharper and more stable boundary than is obtained with solutions containing smaller amounts of impurities.

The following are some examples of the application of the procedure. Fractions of albumin + globulin α, which had been collected from several runs with undiluted serum were dialysed against the above-mentioned phosphate buffer of *p*H 8·04 and run with the large capacity electrode tubes with 400 V. on the apparatus (9·7 V. per cm.). The observed separation of the two boundaries was 4·8 mm. per hr. A compensation of 22 mm. per hr. makes the "apparent" migration velocities of opposite sign approximately equal. By modifications in the voltage and by stopping the compensator clock part of the time small necessary adjustments could be made (as controlled by the optical observation). After 24 hr. the experiment was stopped and the compartments emptied. The albumin boundary was then a little above the top of the upper positive compartment, the globulin boundary (which became very diffuse) was all the way down into the U-tube. One obtained in this way 8 ml. of pure serum albumin, of 2·1 % concentration, and 8 ml. of a solution of globulin α, which however was quite weak (0·2 %).

To obtain larger quantities of globulin α it is better to use an electrodialysed pseudoglobulin solution as starting material. The solution used in the previous publication [Tiselius, 1937, 1] was proved by electrophoretic analysis to contain 85 % of globulin α and 15 % of γ. Fig. 6 shows these two components in a pseudoglobulin solution concentration 1·7 %, after 40 min. at 7·3 V. per cm. and a compensation of 0·79 cm. per hr. in the direction of migration (*p*H = 8·04). Complete separation is effected in about 5 hr. at 400 V. (9·7 V. per cm.). The compensation was 10·5 mm. per hr. at this voltage against the migration. 8 ml. of pure globulin α, of concentration 2·2 %, and 8 ml. of globulin γ, concentration 0·3 %, were obtained from 12·5 ml. pseudoglobulin of concentration

3·0 %. Usually there is some dilution during the migration, especially on the negative side, probably due to the marked changes in buffer concentration taking place in the U-tube in prolonged electrophoresis, on account of the influence of the protein on the transference number of the buffer ions.

Globulin α can also be made easily from serum globulin prepared by precipitation of serum with ammonium sulphate. Since these contain also β the separation takes longer time than when pseudoglobulin is used. Of the two others, globulin γ is much easier to prepare than β. One may obtain it simply from undiluted serum by collecting the layer between the γ- and β-boundaries at the negative side. This component is more heterogeneous than the others, so its boundary is spread out rapidly. It also shows a tendency to become dilute during electrophoresis. Globulin β was prepared in two steps: first β+γ were isolated from serum, then β and γ were separated. In the last operation the γ-boundary was hardly visible, and the separation was run just for the calculated time.

Fig. 6. Photograph of the migrating boundaries in a pseudoglobulin solution.

It is possible to isolate β in one run, thereby avoiding the dilution which is the drawback of repeated operations. Suppose we run serum in the usual way, and arrange so that the β boundary reaches the upper end of the U-tube after a suitable separation time. If now the U-tube is closed, and the layer above, containing albumin, and the albumin+globulin α layers pipetted off with a capillary (which may be inserted through an additional side-tube on the bent connexion tubes to the electrode vessels) and substituted by buffer, the remaining layer willin its upper part contain only albumin, α and β (no γ). If we therefore reverse the current, pure β is left behind, and may be pipetted off. It has not been possible to isolate more than 2 ml. of 0·32 % β in this way.

An apparatus for fractionation of larger volumes is under construction. The investigation of the chemical and solubility properties of the pure fractions, in particular, requires larger amounts of material than can conveniently be prepared with the arrangement described in this paper.

Determination of mobility-pH relationship of the components

The results of the mobility determinations are given in Table II and Fig. 7.

Table II. *Electrophoretic mobilities of the different serum protein fractions at 0° in acetate and phosphate buffer mixtures of varying pH and constant ionic strength 0·1*

		Mobility in cm.² V.⁻¹ sec.⁻¹ × 10⁵			
Buffer	*p*H	Albumin	Glob. α	Glob. β	Glob. γ
Acetate	4·08	+2·96	—	—	—
,,	4·63	+0·13	—	—	—
,,	4·92	−1·36	+0·73	+0·92	+2·22
Phosphate	6·02	−4·60	−3·34	−2·55	+0·01
,,	6·87	−5·90	−4·48	−3·58	−0·99
,,	8·03	−7·15	−6·16	−4·20	−1·51
Isoelectric points		*p*H = 4·64	*p*H = 5·06	*p*H = 5·12	*p*H = 6·0

For accurate mobility measurements one has to take the hydrostatic displacement in the U-tube into account, which counteracts the displacement of

the protein column [*v.* Tiselius, 1930, pp. 44–5]. One may correct for this effect, or it may be eliminated by reducing the free solution area in the electrode tubes by using well-fitting ebonite stoppers. With these one cannot use the present compensation arrangement, which however is very seldom necessary in experiments designed primarily for mobility determination rather than separation. The *p*H values of the buffer solutions used were usually measured at room temperature (18°). Measurements at 0° gave values which differed from these only by 0·01–0·02 *p*H unit.

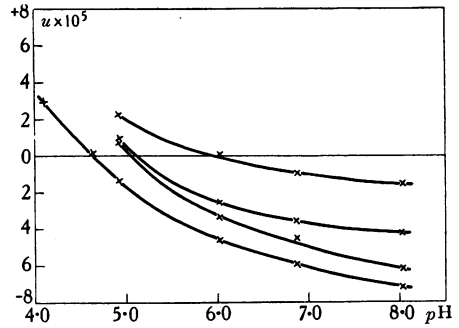

Fig. 7. The mobilities of the isolated serum protein components at varying *p*H and constant ionic strength (0·1).

The temperature of the solution during the experiment is between 0° and 4°. Its exact value may be determined by observing the slight rise in current shortly after starting. However, it is usually quite sufficient to make use of the conductivity at 0° to calculate the potential gradient. If this be done, and if the temperature coefficient of the mobility of the protein is the same as that for the conductivity of the solution, the mobility thus obtained is the exact mobility at 0°, as is easily realized.

A comparison of Fig. 7 with the diagram of the results obtained with unfractioned serum, Fig. 4, clearly demonstrates the fact that the electrophoresis of whole serum essentially is that of a mixture of the distinct components isolated. On the other hand it is seen that the migration velocities in serum under identical conditions are not exactly the same as for each component separately. This may be due to a mutual mobility influence of the protein components or of other constituents present in the original serum but removed in the purification.

The pure globulin components each gave only one boundary, even after prolonged electrophoresis (5 hr. at 300 V.). The β- and γ-globulins gave rather diffuse boundaries, however, whereas the α-boundary remained quite sharp. Evidently the two former components are not quite as homogeneous as the latter.

It should be mentioned that mixtures of serum albumin and globulin α, or globulin α and globulin γ in about equal amounts at $p\mathrm{H} = 8{\cdot}04$, in neither case gave any boundaries other than those corresponding to the two components mixed, with mobilities which were practically unchanged.

The serum albumin always gave a trace of slow material left behind. This component seems to be formed from the albumin on standing or on dialysis. Prolonged electrophoresis also gave an indication of two fast boundaries. It is seen that the isoelectric point is somewhat more acid than has been observed for crystalline serum albumin, *p*H 4·8–4·9 [Tiselius, 1930; Pedersen & Kekwick,

unpublished]. As the present paper was intended to deal chiefly with the globulins, the interesting behaviour of serum albumin will be taken up later.

The measurements on serum globulin in the earlier publication [Tiselius, 1937, 1] refer to mixtures of these components. If the mobilities are reduced from 20° to 0° by multiplying by the ratio of the viscosities, one gets values which agree reasonably well with what would be expected for a mixture of the components studied in the present paper.

The existence of a serum globulin with isoelectric point *p*H 6·1 seems to be of considerable interest, especially as it was found that the antibody belonged to this fraction in the case referred to above.

It cannot be definitely said that these fractions may not be further subdivided. The globulin γ, for example, gave a marked spreading of the boundary. Under such conditions one should use a high concentration to detect traces of other components. Usually the optical method allows observation of components of concentrations down to about 0·02–0·05%, but if the boundaries are much spread out on account of heterogeneity, considerably higher concentrations are required. Imperfections in the windows prevent an otherwise feasible increase in the sensitivity of the optical arrangement. So far the quantities isolated have been insufficient for investigation at high concentration. An optical micro-apparatus is now under construction, requiring only 0·5–1 ml., which will presumably be of much value for further work on the subject.

Sedimentation and diffusion measurements. In the ultracentrifuge the albumin fraction gave a sedimentation constant of $s_{20}=4{\cdot}7\times10^{-13}$, which agrees with previous determinations in this laboratory on crystalline serum albumin ($4{\cdot}5\times10^{-13}$). There was only a trace of material of high sedimentation constant (around $s=17\times10^{-13}$) and nothing of the typical globulin sedimentation ($s=7{\cdot}1$). The electrophoretic material is much more homogeneous in its sedimentation than several times recrystallized albumin, which seems to be quite difficult to purify from globulin. The diffusion of the preparation was not determined.

Of the globulin fractions γ was the most homogeneous and gave only one component of $s_{20}=7{\cdot}0\times10^{-13}$, besides a trace of s_{20} around 18×10^{-13}. No component with albumin sedimentation could be detected. Earlier measurements gave as value for the (unfractionated) serum globulin $7{\cdot}1\times10^{-13}$.[1] The diffusion constant at 20° was $4{\cdot}05\times10^{-7}$ c.g.s., and the diffusion curves also showed a high degree of homogeneity. This and the following diffusion determinations were kindly made by Mr Polson of this laboratory, using Lamm's accurate refractive index method [Lamm & Polson, 1936]. By combination of the sedimentation and the diffusion constants a molecular weight of 165,000 is obtained (unfractioned globulin 167,000). The α- and β-globulins both gave a main fraction of sedimentation constant $s_{20}=6{\cdot}7$–$6{\cdot}8\times10^{-13}$ somewhat lower than for γ. Considerable fractions of lighter and heavier material were present, with $s_{20}=3{\cdot}1\times10^{-13}$ and 18×10^{-13}. The diffusion constants were both $4{\cdot}1\times10^{-7}$ c.g.s. at 20°.

Summing up, we may say, that the albumin and the globulin γ were more homogeneous in sedimentation than material prepared by the common precipitation methods. The two other fractions were rather less homogeneous. It must be emphasized, however, that according to recent results in this laboratory by Pedersen [1936] the sedimentation constant of serum globulin cannot be considered to be an unequivocal characteristic of this protein, as in presence of other substances, for example serum albumin, it is partially transformed into

[1] *Tabul. biol., Berl.*, Ed. W. Junk, **5**, 352–3 (1935–6).

a form characterized by a lower sedimentation of the same magnitude as that of albumin.

The relationship of the components described to "euglobulin" and "pseudoglobulin". It has already been mentioned that serum protein fractions prepared by ammonium sulphate precipitation were found to contain all the components described, but in greatly varying amounts, so that the fractions most easily precipitated contain mainly γ and β and smaller amounts of α and albumin, whereas the proteins precipitated above 55 % saturation with ammonium sulphate contained about 75 % albumin and only 25 % of the globulins. Serum globulin, reprecipitated 3 times with 55 % ammonium sulphate, dissolved in 5 % sodium chloride, dialysed and electrodialysed, consisted of 75–85 % of globulin α, the rest of a slow component, probably γ. Euglobulin (serum globulin I in the previous publication, Tiselius [1937, 1] contained more β and γ, and less α. Very probably therefore the solubility differences between pseudo- and euglobulin depend upon the different solubilities of the α and the β, γ fractions.

General remarks. The investigations with the main object of dividing serum into its fractions are too numerous to be referred to here. It may be sufficient to point to the fundamental work of Sørensen and his collaborators, who mainly used ammonium sulphate precipitation, the classical method of protein fractionation. That much valuable information may still be gained along this line is shown for example by the recent findings of Hewitt [1936; 1937] regarding the fractionation of serum albumin into a carbohydrate-free and a carbohydrate-rich component. Especially through the work of Svedberg, there has grown a recognition of the necessity of studying directly protein systems from the point of view of homogeneity in solution, since information is thus gained as to the actual condition of the components present, without the risk of changing this condition by more or less violent methods used for fractionation. The ultracentrifuge investigations of Svedberg & Sjögren [1930], v. Mutzenbecher [1933] McFarlane [1935], Pedersen [1936], Heidelberger & Pedersen [1937], have thus given much new information about the serum proteins. Besides ultracentrifuging (and possibly ultrafiltration and diffusion) electrophoresis seems to be an especially suitable method for work of this kind. In his well-known work "Colloidal Solution. The Globulins" Sir William Hardy [1905], who was the first to observe the electrophoresis of proteins, studied serum with a migration apparatus, and it is quite evident that he realized the importance of this method for homogeneity studies. Progress in the work with serum by means of electrophoresis has been slow, however, as the experimental difficulties are especially great, on account of the high conductivity. In recent years many interesting contributions have been made. Theorell [1930] showed that albumin and globulin migrate at different rates in serum, by analysing the contents of the U-tube by precipitation methods after sending current through. A similar method was used by Bennhold [1932]. McFarlane [1935], using Theorell's apparatus, was able to isolate sufficient amounts of albumin and globulin of human serum for determinations in the ultracentrifuge and from his results concluded that the method gave very homogeneous preparations and also suggested that this procedure may be valuable for isolation of non-crystallizing albumins. Kylin [1934] and especially Grönwall [1935] also made observations on the electrophoresis of serum. The latter author, using a modification of Bennhold's apparatus observed, by the aid of a Tyndall beam, 5–9 sharp boundaries in the positive limb of the U-tube, when subjecting undiluted sera to prolonged electrophoresis (36–48 hr.) against a Ringer solution. The number of boundaries varied for different animals and even for different individuals. The conditions of these experiments are not

sufficient to guarantee undisturbed migration. The facts that the boundaries are visible only in one limb and that the load on the apparatus was very high suggest that convections may have interfered. It has been emphasized above that convections tend to develop very sharp "false" boundaries. This occurs almost invariably if some of the components are allowed to migrate through the bottom of the U-tube, as seems to have been the case in these experiments. Nevertheless it seems possible that some of the boundaries observed by Grönwall may correspond to components described in the present paper.

The heterogeneity in electrophoresis of serum globulin was observed by the author [1930] and special attention was given to the fact that the preparations studied were nevertheless homogeneous in ultracentrifugal sedimentation. The existence of three electrochemically quite different serum globulins with approximately the same molecular weights has been established above. One might perhaps suggest as an explanation that the three globulins are nearly identical, and the differences in charge are caused by constituents of low weight, bound to the identical main constituent. If this be the case, it should be emphasized that the charge-determining factor must be firmly bound, since otherwise an isolation of single components would have been impossible. Furthermore the additive behaviour on mixing pure components would be hard to explain, since one would expect the different forms to be in equilibrium, if there is loose combination.

The identity of the molecular weights is, however, by no means an indication of chemical identity, as is quite clear from the large number of ultracentrifugal data now collected by Svedberg and his co-workers. There is even a tendency for the molecular weights to group themselves into classes, each containing proteins of widely different chemical properties but of nearly the same size. The author is rather inclined to believe that this is the case also with the serum globulins, especially as the ultraviolet absorption is markedly different. Further investigation of the chemical properties of these substances will probably throw more light on that question.

In conclusion a few remarks should be made about the significance of the results obtained by electrophoretic analysis of "biocolloid systems". It is characteristic of this method that we obtain information about the components of a system subjected to a very mild agent, and this information therefore refers closely to the conditions prevailing in the original system. This does not necessarily mean that our components represent chemical individuals. It was mentioned above that bilirubin migrates with the albumin and cannot be separated from this by electrophoresis. On the other hand we know that by many recrystallizations a colourless albumin may be produced. Evidently ammonium sulphate precipitation to a certain degree breaks down the original complex. The gradual application of less mild procedures would seem to be the proper method of approaching the structure of such systems. It should be emphasized that the electrophoretic analysis lends itself also to such a gradual increase in the violence of attack: by work at extreme *p*H values or salt concentrations, by addition of enzymes or other substances known to cause a breakdown, new systems are made which are equally well suited for an analysis, along the lines demonstrated in this paper.

Summary

A procedure has been worked out by which mixtures containing several components (proteins and other high molecular compounds) may be studied from the point of view of chemical homogeneity and also separated into their main constituents, by means of an improved electrophoresis apparatus. The improve-

ments have made possible a considerable increase in the potential gradient in the electrophoresis tube and therefore a more rapid and complete separation. The separation capacity of the apparatus is made use of to its full extent by a special compensation device. Optical observation of the boundaries can be made by a sensitive method (Toepler's Schlieren method) and may also allow a quantitative estimation of the amounts of the components.

With the aid of this method serum, as well as solutions of serum globulin, were found to contain several distinctly different components, which could be completely separated. Accordingly serum is not a more or less continuous mixture, but contains well-defined protein fractions: albumin and three globulins α, β and γ. The globulins have approximately the same molecular weight, but quite different electrochemical properties: e.g. the isoelectric point of globulin γ is at $p\mathrm{H} = 6{\cdot}0$, instead of 5·1 for the globulins α and β. In addition the mobilities are quite different, especially at alkaline reactions.

Investigation of a highly potent anti-egg albumin serum from rabbit showed that the antibody function migrated with the γ-globulin fraction only. By isolation of this fraction a considerable concentration of specifically precipitable protein could be obtained.

The significance of the results and the method is discussed, and the wide applicability of the latter is emphasized.

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